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MICROPROPAGATION

In vitro propagation of ten threatened species of *Mammillaria* (Cactaceae)

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Abstract *Mammillaria* species are the most numerous within Cactaceae family, and some of them are threatened with extinction as a result of human activities. In this work, results of *in vitro* propagation are presented for ten *Mammillaria* species, testing 20 combinations of indole-3-acetic acid (IAA) and kinetin. Best results on shoot formation were obtained using kinetin at two levels: 27.9 and 46.5 μM. All IAA levels tested were able to induce *de novo* shoot formation in *M. bocasana*, *M. densispina*, *M. hahniana*, *M. hutchisoniana*, *M. orcutii*, *M. pectinifera*, *M. perbella*, *M. picta*, *M. rhodantha*, and *M. zephyranthoides*. Depending on the IAA level tested, four responding groups were observed concerning their highest shoot-formation number. For all species, the highest average of shoot formation was achieved with 5.7:46.5 or 11.4:46.5 μM IAA/kinetin, yielding 4.8 and 4.7 shoots per explant, respectively, in 60 d. Rooting of regenerated shoots was achieved by leaving the explants in their shoot-induction medium or transferring them to half-strength MS medium. Hardening of regenerated plants was successfully achieved by planting them in peat moss substrate after a desiccation treatment at room temperature for 3 d.

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Introduction

The Cactaceae family excels among the American flora with more than 1,500 species. Cacti thrive through the American continent, including tropical and subtropical areas, but most of them are found on arid lands. They frequently survive as endemic species with varying levels of threatening status.

Among the Cactaceae, the genus *Mammillaria* is the largest one, constituting one of the most popular group of cacti for marketing. This genus exhibits a great variety of stem forms, flower color, and generally small size, factors for which many of the species of this group are threatened with extinction as a result of extensive harvesting in their natural habitat. According to Rubluo (1997), most of the species of *Mammillaria* have their center of genetic diversity in Mexico, growing from the coast to the mountains but mostly in the arid and semiarid regions. Hunt (1992) has catalogued 392 *Mammillaria* species, most of them (373) from Mexico, albeit Guzmán et al. (2003) filed 1,025, just for Mexican *Mammillaria*, including their subspecies. Many of the species are listed under the Mexican Official Norm (Norma Oficial Mexicana NOM-059-ECOL-2001 2002) as endangered species, and genus *Mammillaria* has been considered a severely threatened group (Rubluo 1997; Glass 1998). *Mammillaria* species are mainly used as ornamental plants, but medicinal and industrial uses have been pointed out by Rubluo (1997). Gómez-Hinostrosa and Hernández (2000) have advised that, because the ecological disturbance is growing and is nearing everyday to the apparently non-threatened species

habitats, it is necessary to preserve all Cactaceae, regardless of their status.

Several authors have applied tissue culture techniques as an alternative to preserve the threatened Cactaceae (Vyskot and Jára 1984; Rubluo et al. 1993, 2002; Perez-Molphe et al. 1998; Giusti et al. 2002; Zavala-Hurtado and Valverde 2003). So far, the *in vitro* culture of *Mammillaria* cacti has been reported for 33 species; therefore, most of them need to be *in vitro* propagated and saved from extinction. Different combinations and concentrations of plant growth regulators (PGR) for *in vitro* culture of *Mammillaria* have been tested; e.g., Kolar et al. (1976) regenerated shoots from explants of *M. woodsii* using the MS medium (Murashige and Skoog 1962) with 11.4 μM IAA and 9.3 μM kinetin; Starling and Dodds (1983) utilized only benzyladenine (BA) as a PGR to propagate *Mammillaria glasii*; Martínez-Vazquez and Rubluo (1989) used 0.44 μM BA combined with either 0.54 or 0.5 μM 1-naphthaleneacetic acid (NAA) for *in vitro* culture of *M. san-angelensis*; Rubluo (1997) recommended low dosages of BA alone (0.44 μM), or combination of 0.44 μM BA and 0.05 μM NAA for *Mammillaria* propagation; Elías-Rocha et al. (1998) propagated *M. candida* by tissue culture techniques, finding best results using kinetin 23.2 μM alone or in combination with NAA 0.054 μM; Pérez-Molphe et al. (1998) reported the use of 4.4 μM BA alone or combined with either 0.54 or 0.5 μM NAA for several *Mammillaria* species.

The *in vitro* responses of ten *Mammillaria* species to 20 combinations of IAA and kinetin are described in this work. Eight out of 10 *Mammillaria* species studied have not been previously reported as cultured *in vitro* (*M. densispina*, *M. hahniana*, *M. hutchisoniana*, *M. orcutii*, *M. perbella*, *M. picta*, *M. rhodantha*, and *M. zephyranthoides*). *M. bocasana* and *M. pectinifera* are species previously reported to be cultured *in vitro* (Pasqual and Hoshika 1992; Giusti et al. 2002), albeit their reported maximum propagation rates were 1.8 and 3.5 shoots per explant, respectively. These species were included in this work with the aim to improve those previous results. The reported rates for *M. bocasana* and *M. pectinifera* (Pasqual and Hoshika 1992; Giusti et al. 2002) were surpassed with the protocol described in this work.

Materials and Methods

Plant material. Plant material (young plants 2–4 y old) was obtained from diverse collectors and Cactaceae nurseries. At least one individual plant of the following species was obtained in early 2001: *M. bocasana*, *M. densispina*, *M. hahniana*, *M. hutchisoniana*, *M. orcutii*, *M. pectinifera*, *M. picta*, *M. perbella*, *M. rhodantha*, and *M. zephyranthoides.* Plants were initially fungicide treated and maintained during 1 y in polyethylene greenhouse without temperature control. Briefly, plants were immersed in a fungicide solution, using 1 ml l^{-1} of each one of the following fungicides: Previcur N, Bayer CropScience S. A., Santa Fe de Bogota, Colombia; Derosal 500D, Bayer CropScience, Lyon, France; Buzan 30 WB, Buckman Laboratories S.A., Cuernavaca, Mexico). After greenhouse-treatment, 2–5 g sections from the upper portion of each plant were taken. Plant sections were washed with distilled sterile water and dried for 5 h in a laminar flow chamber. Explants were externally disinfected with 70% ethanol for 60 s, followed by a second disinfection for 20 min with 20% vegetable sanitizer (Microdyn, Roland de México S. A., Cuernavaca, Mexico) containing 0.048% silver colloids. A third treatment with 20% (*v*/*v*) commercial sodium hypochlorite containing 0.1% (*v*/*v*) Tween 20 (Sigma-Aldrich Química S. A., Cuernavaca, Mexico) was applied for 20 min. Explants were rinsed five times in sterile distilled water.

In vitro culture establishment. After external disinfection of explants, the whitish tissue was discarded, and the tissue sections were divided using a scalpel into explants bearing at least three areoles. These explants were placed in 120-ml food jars containing 20 ml of MS medium plus 3% sucrose. pH was adjusted to 5.7–5.8 before 8 g l^{-1} of agar (Sigma-Aldrich Química S. A.) were added. The culture medium was supplemented with 13.9 μM kinetin and 2.8 μM IAA, as a preliminary treatment for *in vitro* establishment of different *Mammillaria* species. The explants were cultured under a 16 h photoperiod (daylight fluorescent lamps, 35 μMol m^{-2} s^{-1}) at 25±2°C.

Micropropagation and evaluation of explant responses. Each species was subjected to a factorial experiment, employing 200 explants derived from the preliminary multiplication step. The explants, depending on species, were grown over a period of 6 to 18 mo. during the years 2002–2003, and the factorial experiments were carried out during the years 2003 and 2004. Once enough plant material from each species was obtained, its respective factorial experiment was established, consisting of 20 treatments with one explant per bottle, with ten replicates. The factorial design consisted of four concentrations of IAA (0, 5.7, 11.4, and 22.8 μM,) and 5 of kinetin (0, 4.6, 13.9, 27.9, and 46.5 μM), with subcultures every 30 d. Each experiment was repeated twice, and results were reported as averages of ten repetitions and two experiments.

Final evaluation of the explant response in each experiment was performed after 60 d of culture, assessing the number of buds by explant for each treatment. Results were subjected to analysis of variance. To discriminate among the mean values, Fisher's least significant difference (LSD) with α=0.05 was applied using the Statgraphics program.

Rooting and hardening. Rooting of *in vitro* propagated plants was achieved in two ways: (1) Some shoots rooted directly in their respective regeneration medium; and (2) plants that did not root in their regeneration medium were subsequently transferred onto half-strength MS medium without PGR to promote rooting. Rooted cacti, with a stem of 1 or 2 cm in length were removed from culture flasks and washed in tap water to eliminate the residual agar.

These cacti were desiccated for 3 d at room temperature. Afterward, they were externally treated with 2 g l^{-1} Captan 50 PH (Rhone-Poulenc Agro S. A., Cuernavaca, Mexico) plus 1 ml l^{-1} Buzan 30WB (Buckman Laboratories, S. A., Cuernavaca, Mexico) to avoid fungi growth. The basal part of the regenerated cacti plants was immersed during 20 min in a solution containing 492 μM IBA (Sigma-Aldrich Química S. A.) to promote rooting, planted in pots (7.6-cm diameter) containing sterilized peat moss substrate, and placed under a polyethylene greenhouse without temperature control.

Results

The combination of disinfection treatments using fungicides, commercial sanitizer, and sodium hypochlorite provided a reliable system to remove most of explant's surface contaminant microorganisms. However, 80% of explants failed the *in vitro* culture because of extensive oxidation, contamination, and/or necrosis. On average, 20% of explants for all *Mammillaria* species were successfully *in vitro* established.

The general organogenic response trend of all species to the 20 different concentrations treatments indicated that 5.7:46.5 μM and 11.4:46.5 μM IAA/kinetin induced the highest average responses, with 4.7 and 4.8 shoots per explant, respectively (Table 1). Direct and indirect (callus growth) organogenesis was observed on *Mammillaria in vitro* regeneration. These phenomena were dependent on *Mammillaria* species and auxin concentration in the culture media; e.g., higher IAA levels induced more callus growth. In *M. bocasana*, *M. densispina*, *M. pectinifera*, *M. picta*, *M. perbella*, and *M. zephyranthoides*, both organogenic systems were observed, whereas in *M. rhodantha*, *M. hahniana*, *M. hutchisoniana*, and *M. orcutii*, only direct organogenesis was detected (Fig. 1*A*).

Shoot proliferation. IAA and kinetin induced small calli around the basal part of explants. These calli were observed after 3 wk of culture. Likewise, new offshoots started to grow directly from mammillae's axils. When the medium included only IAA, some shoots sporadically regenerated from callus surface (data not shown). On media without cytokinins, a low frequency adventitious shoot formation was recorded, whereas the major responses, measured as regenerated shoot number, occurred in the highest concentrations of kinetin (Table 1).

In vitro shoot formation rate had a positive correlation with the cytokinin concentration; i.e., higher cytokinin levels (27.8 and 46.5 μM) increased shoot formation. Likewise, *in vitro* explant response was dependent on the species and on the auxin (IAA) levels. Four responding species groups related to each concentration of IAA (0, 5.7, 11.4, and 28.8 μM) were observed (Table 1).

The first group of species with the highest response in the absence of IAA included *M. bocasana*, *M. hutchisoniana*, and *M. rhodantha*. The average shoot formations of these species were four, five, and five shoots per explant, respectively.

The second group, constituted by *M. orcutii* and *M. perbella*, responded better in the presence of 5.7 μM IAA in combination with 46.5 μM kinetin. The shoot formation averages for *M. orcutii* and *M. perbella* were 17 and 8 shoots per explant, respectively.

The third group included *M. picta* and *M. zephyranthoides*. Using 11.4 μM IAA in combination with either 46.5 or 27.8 μM kinetin, an average number of six and eight shoots per explant was observed, respectively.

The fourth group was formed by *M. densispina*, *M. hahniana*, and *M. pectinifera*. These *Mammillaria* species produced an average number of five, two, and four shoots per explant, respectively, after 60 d in culture on media with 22.8 μM IAA, in combination with either 46.5 or 27.8 μM kinetin.

Shoot rooting and plant hardening. In general, the rooting of *Mammillaria* shoots took place on the same medium on which they were induced; however, exceptions were *M. orcutii* and *M. perbella*, which needed a subculture on a half-strength MS medium. Rooted plants of *Mammillaria hutchisoniana* are depicted on Fig. 1*B*.

Despite using sterile soil and several fungicides, it was necessary to treat the soil with a fungicide mixture solution (1 ml l^{-1} of each of the following fungicides: Previcur N, Bayer CropScience S. A.; Derosal 500D, Bayer CropScience; Buzan 30 WB, Buckman Laboratories, S. A.) to avoid plant infection. As the regenerated cacti plants transferred to soil had severe root rot problems, it was decided to promote plant hardening by leaving the cacti dehydrate for 3 d at room temperature and under shed conditions just after removing them from *in vitro* jars. Later on, the basal part of the treated plants was immersed in a 492-μM IBA solution to promote rooting and treated with fungicides to prevent root rot problems. Plants restarted their normal development 3 to 4 wk later (Fig. 1*B*). The following survival data, after being transplanted to soil, were obtained for each *Mammillaria* species: *M. bocasana*, 93.9%; *M. densispina*, 87.5%; *M. hahniana*, 96.5%; *M. hutchisoniana*, 92.0%; *M. orcutii*, 84.5%; *M. pectinifera*, 90.0%; *M. picta*, 86.0%; *M. perbella*, 92.2%; *M. rhodantha*, 92.5%; and *M. zephyranthoides*, 88.2 %.

Table 1. Average number of axillary and adventitious shoots in explants of ten *Mammillaria* species as a response to different IAA/kinetin combinations after 60 d of *in vitro* culture

IAA (μM)	Kinetin (μM)	*M. bocasana*	*M. densispina*	*M. hahniana*	*M. hutchinsoniana*	*M. orcutii*	*M. pectinifera*	*M. picta*	*M. perbella*	*M. rhodantha*	*M. zephyranthoides*
0	0	1.1 c	1.0 c	1.0 c	0.6 d	1.0 cd	1.1 d	0.6 d	1.1 f	1.4 bc	1.0 e
0	4.6	1.8 bc	1.0 c	0.9 c	0.7 d	1.4 cd	1.1 d	1.8 bcd	1.0 f	2.4 bc	1.1 e
0	13.94	2.0 bc	1.0 c	1.0 c	1.9 bcd	1.0 cd	2.2 bcd	3.0 bcd	2.8 cdef	1.8 bc	1.2 de
0	27.88	2.3 b	3.6 ab	0.6 c	2.9 abcd	0.8 d	4.3 ab	3.6 abcd	5.7 abc	5.4 a	3.0 cd
0	46.5	3.8 a	4.6 ab	1.6 abc	5.2 a	1.6 cd	2.6 abcd	1.2 cd	5.6 abc	4.2 ab	3.4 bc
5.7	0	2.2 bc	1.0 c	1.0 c	0.5 d	1.0 cd	1.5 cd	1.4 cd	1.0 f	1.6 bc	1.0 e
5.7	4.6	1.5 bc	1.0 c	1.0 c	0.9 cd	1.0 cd	2.2 bcd	1.0 d	1.3 f	1.2 c	1.0 e
5.7	13.94	1.4 bc	1.4 c	1.0 c	4.0 ab	2.4 cd	2.3 abcd	1.4 cd	3.4 cdef	1.4 bc	1.1 e
5.7	27.88	1.6 bc	2.8 bc	0.8 c	4.0 ab	11.4 b	3.4 abc	1.4 cd	4.5 bcde	1.0 c	2.0 cde
5.7	46.5	1.5 bc	3.6 ab	1.6 abc	4.1 ab	17.4 a	2.6 abcd	2.6 bcd	7.9 a	3.2 abc	3.3 bc
11.42	0	1.4 bc	1.0 c	0.8 c	1.3 cd	1.0 cd	1.6 cd	1.0 d	2.0 ef	1.2 c	1.0 e
11.42	4.6	1.7 bc	1.0 c	1.0 c	1.7 bcd	1.0 cd	1.1 d	2.4 bcd	2.5 def	3.0 abc	1.1 e
11.42	13.94	1.4 bc	1.4 c	1.0 c	3.5 abcd	3.0 cd	1.4 cd	2.2 bcd	4.7 bcde	1.6 bc	1.1 e
11.42	27.88	2.0 bc	5.0 a	0.8 c	3.5 abcd	6.0 c	2.2 bcd	3.6 abcd	3.7 bcdef	1.2 c	7.6 a
11.42	46.5	1.4 bc	4.1 ab	2.2 ab	2.8 abcd	12.4 ab	2.6 abcd	6.4 a	6.6 ab	3.2 abc	5.0 b
22.83	0	1.5 bc	1.0 c	1.0 c	1.2 cd	1.0 cd	1.1 d	1.6 bcd	1.4 f	1.2 c	1.0 e
22.83	4.6	1.9 bc	1.0 c	1.0 c	0.7 d	1.4 cd	1.2 cd	1.0 d	1.1 f	1.4 bc	1.0 e
22.83	13.94	1.3 c	1.4 c	1.2 bc	2.8 abcd	1.2 cd	2.8 abcd	2.5 bcd	1.4 f	1.8 bc	1.2 de
22.83	27.88	2.0 bc	4.1 ab	1.4 abc	1.7 bcd	5.2 cd	4.5 a	4.4 abc	1.9 ef	2.2 bc	1.9 cde
22.83	46.5	1.7 bc	4.8 a	2.4 a	0.9 cd	11.6 b	2.3 abcd	4.8 ab	5.1 abcd	3.4 abc	3.2 bc

Values within *columns* with the same *letter* are not significantly different from each other at 5% level by LSD (Fisher test).

Discussion

This study was focused on *in vitro* micropropagation of *Mammillaria* species, albeit combining it with *in vivo* conservation of regenerated plants. We assume, as many micropropagators of these species have claimed (Vyskot and Jára 1984; Clayton et al. 1990; Rubluo 1997; Perez-Molphe et al. 1998; Papafotiou et al. 2001; Giusti et al. 2002), that conservation of threatened species should be supported by tissue culture biotechnology.

The *in vivo* conservation of Cactaceae requires an adequate space and extensive labor force. However, *in vitro* techniques offer the unique opportunity of maintenance of plant materials under aseptic and controlled conditions, avoiding the problems of diseases, pests, and environmental changes that could affect the plant collections. Clayton et al. (1990) and Rubluo (1997) claimed that it is important to develop particular protocols for each plant species. However, that statement is partially true, as in the present study, different *Mammillaria* species cultured *in vitro* had common plant growth regulator requirements. For example, all *Mammillaria* species required the presence of similar or equal concentrations of cytokinin to induce their highest shoot number formation. *M. bocasana*, *M. hutchisoniana*,

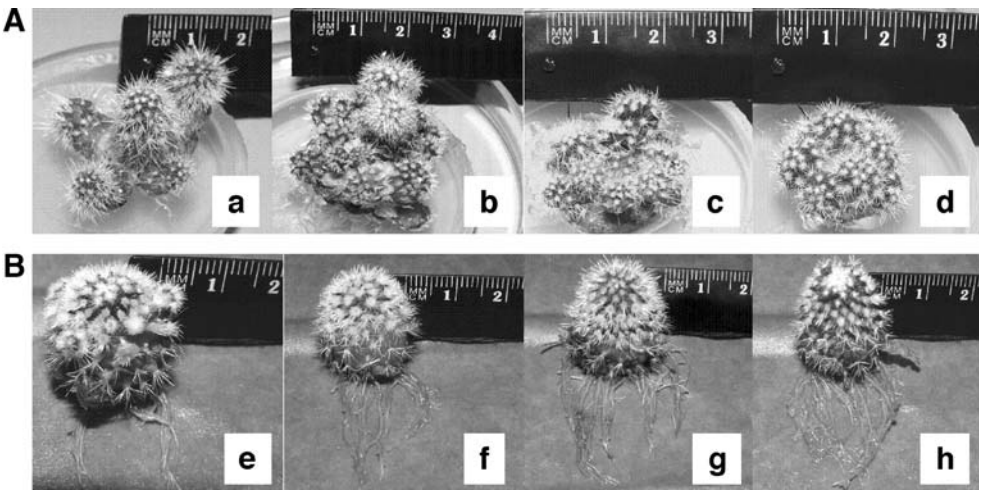

Figure 1. Micropropagation (*A*) and rooting (*B*) of *Mammillaria hutchisoniana* on different IAA/kinetin concentrations: (*a*) 0 μ*M* IAA+46.5 μ*M* kinetin, (*b*) 5.7 μ*M* IAA+46.5 μ*M* kinetin, (*c*) 11.4 μ*M* IAA+13.9 μ*M* kinetin, and (*d*) 22.8 μ*M* IAA+13.9 μ*M* kinetin, (*e*) 0 μ*M* IAA+46.5 μ*M* kinetin, (*f*) 5.7 μ*M* IAA+46.5 μ*M* kinetin, (*g*) 11.4 μ*M* IAA+13.9 μ*M* kinetin, and (*h*) 22.8 μ*M* IAA+13.9 μ*M* kinetin.

M. hahniana, *M. picta*, *M. orcutii*, and *M. perbella* needed the same concentration of kinetin (46.5 μM) to induce their maximum organogenic response. The other four species, *M. pectinifera*, *M. densispina*, *M. rhodantha*, and *M. zephyranthoides*, also required a similar concentration of kinetin (27.9 μM) to induce their highest shoot number formation (Table 1).

All *Mammillaria* species had very poor responses when kinetin was absent from the culture medium, suggesting that cytokinins are essential for shoot organogenesis. The species *M. bocasana*, *M. hutchisoniana*, and *M. rhodantha* had their highest shoot number formation in the presence of kinetin alone. Likewise, *M. orcutii* and *M. perbella* exhibited the highest shoot number on a medium with 5.7 μM IAA in combination with 46.5 μM kinetin. *M. densispina* and *M. zephyranthoides*, on the other hand, had the best response after being treated with 11.4 μM IAA combined with 27.9 μM kinetin, and *M. picta* provided the best response with 11.4 μM IAA combined with 46.5 μM kinetin. *M. densispina* and *M. hahniana* were found to respond better in the presence of 22.8 μM IAA in combination with 46.5 μM kinetin, and *M. pectinifera* produced the highest shoot number after being treated with 22.8 μM IAA plus 27.88 μM kinetin (Table 1).

Although the results described herein are similar to those previously reported (Vyskot and Jára 1984; Rubluo et al. 1993; Rubluo 1997; Pérez Molphe et al. 1998), two main advanced differences should be depicted: Firstly, eight *Mammillaria* species, which had not been previously reported, were included in the present study; and secondly, the micropropagation rates for *M. bocasana* and *M. pectinifera* achieved in the present work were superior to those previously reported (Pasqual y Hoshika 1992; Giusti et al. 2002).

According to Vyskot and Jára (1984), Rubluo et al. (1993), Rubluo (1997), and Pérez Molphe et al. (1998), the *in vitro* micropropagation of different *Mammillaria* species take place on a medium with cytokinin concentrations from 4.4 to 46.4 μM. Nevertheless, there are also reports on *in vitro* propagation of *Mammillaria* species either in the absence of PGR or in the presence of auxin alone (Papafotiou et al. 2001). For example, Papafotiou et al. (2001) reported the propagation of *M. elongata* in the absence of PGR or in the presence of 0.54 μM NAA. Rubluo et al. (2002) reported the propagation of *M. san-angelensis* on culture medium with auxin alone, achieving the maximum proliferation rate at 34.2 μM IAA. Therefore, the *in vitro* culture of *Mamillaria* species has a set of coincidences and differences related to the amount and types of PGRs needed for their micropropagation.

M. bocasana and *M. pectinifera* were included in the present study with the aim of improving their multiplication rates. *M. bocasana* had previously been studied by Pasqual and Hoshika (1992), who reported a low shoot formation (no quantitative data were reported). In the present study, a propagation rate of 3.8 shoots per explant was achieved. A proliferation rate of 4.5 shoots per explant was attained for *M. pectinifera* using 22.8:27.9 μM IAA/kinetin (Table 1), surpassing slightly the rates described by Giusti et al. (2002). These authors achieved a proliferation rate of 1.5 shoots per explant in the presence of 22.2 μM BA. Additionally, they reached a proliferation rate of 3.5 shoots per explant by means of 2.3 μM 1-phenyl-3-(1-2-3-thiadiazol-5-YL) urea (TDZ); however, hyperhydricity (vitrification) problems were reported. Hyperhydricity was not observed in any *Mammillaria* explant under the protocols described herein.

Several authors have considered that hardening of cacti plants derived from *in vitro* cultures is the most common problem (Rodriguez-Garay and Rubluo 1992; Rubluo et al. 1993). Nevertheless, other authors have considered that hardening is relatively easy (Perez-Molphe et al. 1998; Giusti et al. 2002). At the beginning of the present work, plant establishment in soil was carried out in a sterile substrate and treated with fungicides like Benomyl and/or Captan. Despite that, root rot appeared on cacti, and almost all plants had perished in a few days. Hence, it was decided to subject the *in vitro* plants to hardening for 3 d at room conditions and under the shade without watering of either substrate or cacti. Withholding the watering led to the loss of water excess accumulated during the *in vitro* culture. Subsequently, the basal part of semi-desiccated plants was immersed during 20 min in a 492 μM IBA solution and fungicide products for 20 min. After this treatment, almost all plants reassumed rooting and growth in about 30 d (Fig. 1*B*).

So far, this simple method of hardening cacti plants from *in vitro* culture has not been reported before, and although very simple, it provides a good plant establishment in soil. It might be possible to suggest that the dehydration period removes the water excess from the tissue, avoiding the root rot. This is in agreement with Malda et al. (1999), who have considered that cacti have a proficient capability to rehydrate after culture *in vitro*. Moreover, Perez-Molphe et al. (1998) and Giusti et al. (2002) have also reported no problems with hardening of *in vitro* regenerated cacti.

The present study is an important contribution to the conservation of extinction-threatened Cactaceae species. We have established an easy and reliable micropropagation protocol for *Mammillaria* species and we have also developed a simple, inexpensive and reliable system for cacti hardening, which might be applied to other *Mammillaria* species or Cactaceae plants.

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References

Clayton, P.W; Hubstenberger, J.F; Phillips, G.C; Butler-Nance, S. Micropropagation of members of the *Cactaceae subtribe* Cactinae. J. Am. Soc. Hortic. Sci. 115:337–343; 1990.

Elías-Rocha, MA; Santos-Díaz, MD; Arredondo-Gómez, A. Propagation of *Mammillaria candida* (Cactaceae) by tissue culture techniques. Haseltonia 6:96–101; 1998.

Giusti, P.; Vitti, D.; Fiocchetti, F.; Colla, G.; Saccardo, F.; Tucci, M. *In vitro* propagation of three endangered cactus species. Sci. Hortic. 95:319–332; 2002.

Glass, E.C. Identification guide to threatened cacti of Mexico. Fideicomiso Fondo para la Biodiversidad. 206 pp. 1998.

Gómez-Hinostrosa, C.; Hernández, H.M. Diversity, geographical distribution and conservation of Cactaceae in the Mier and Noriega region, México. Biodivers. Conserv. 9:403–418; 2000.

Guzmán, U.; Arias, S.; Dávila, P. Catálogo de cactáceas Mexicanas. Universidad Nacional Autónoma de México. CONABIO. 315 pp. 2003.

Hunt, D. CITES Cactaceae checklist. Royal botanic gardens Kew, London, UK. 190 pp. 1992.

Kolar, Z.; Bartek, J.; Viskot, B. Vegetative propagation of the cactus *Mammillaria woodsii* through tissue cultures. Experientia 32:668–669; 1976.

Malda, G.; Suzan, H.; Backhaus, R. *In vitro* culture as a potential method for the conservation of endangered plants possessing crassulacean acid metabolism. Sci. Hortic. 81:71–87; 1999.

Martínez-Vazquez, O.; Rubluo, A. *In vitro* mass propagation of the near-extinct *Mammillaria san-angelensis* Sánchez-Mejorada. J. Hortic. Sci. 64:99–105; 1989.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissues cultures. Physiol. Plant. 15:473–479; 1962.

Norma Oficial Mexicana NOM-059-ECOL-2001, 2002. Protección ambiental de especies nativas de México de flora y fauna silvestres. Categorías de riesgo específicas para su inclusión, exclusión o cambio-lista de especies en riesgo. Diario Oficial de la Federación. Segunda Sección (6 de marzo de 2002), México, pp 1–81; 2002.

Papafotiou, M. ; Balostis, G.N. ; Louka, P.T. ; Chronopoulos, J. *In vitro* plant regeneration of *Mammillaria elongate* normal and cristate forms. Plant Cell Tissue Organ Cult. 65:163–167; 2001.

Pasqual, M.; Hoshika, E. Efeitos do ácido naftaleno acético e 6-benzilaminopurina sobre a proliferação *in vitro* de cactos *Gymnocalicium buldiamur* L. e *Mammillaria bocasana* L. Pesqui. Agropecu. Bras. 27:289–593; 1992.

Perez-Molphe, E.; Pérez-Reyes, M.E.; Villalobos-Amador, E.; Meza-Rangel E.; Morones-Ruíz, L.R.; Lizalde-Viramontes, H.J. Micropropagation of 21 species of Mexican cacti by axillary proliferation. *In Vitro* Cell. Dev. Biol. Plant 34:131–135; 1998.

Rodríguez-Garay, B., Rubluo, A. *In vitro* morphogenetic responses of the endangered cactus *Aztekium ritteri* (Boedeker). Cact. Suc. J. (USA) 64:116–119; 1992.

Rubluo, A. 1997. Micropropagation of *Mammillaria* species (Cactaceae). In: Bajaj, Y. P. S., ed. Biotechnology in agriculture and forestry, 40. High tech and micropropagation. VI. Springer: Heidelberg; 1997:191–205.

Rubluo, A.; Chavez, V.; Martínez, A.P.; Martínez-Vazquez, O. Strategies for the recovery of endangered orchids and cacti through *in vitro* culture. Biol. Conserv. 63:163–169; 1993.

Rubluo A.; Marín-Hernández, T.; Duval, K.; Vargas, A.; Marquez-Guzmán, J. Auxin induced morphogenetic responses in long-term *in vitro* subcultured *Mammillaria san-angelensis* Sánchez-Mejorada (Cactaceae). Sci. Hortic. 95:341–349; 2002.

Starling, R.J.; Dodds, J.H. Tissue culture propagation of cacti and other succulents. Bradleya 1:84–90; 1983.

Vyskot, B.; Jára, Z. Clonal propagation of cacti through axillary buds *in vitro*. J. Hortic. Sci. 59:449–452; 1984.

Zavala-Hurtado, J.A.; Valverde P.L. Habitat restriction in *Mammillaria pectinifera*, a threatened endemic Mexican cactus. J. Veg. Sci. 14:891–898; 2003.